

OBSERVATIONS ON AN ATP-SENSITIVE PROTEIN SYSTEM  
FROM THE PLASMODIA OF A MYXOMYCETE\*

By PAUL O. P. TS'O, JAMES BONNER, LUTHER EGGMAN,  
AND JEROME VINOGRAD

(From the Kerckhoff Laboratories of Biology and the Gates and Crellin Laboratories  
of Chemistry, ‡ California Institute of Technology, Pasadena)

(Received for publication, June 13, 1955)

The plasmodia of the slime mold, *Physarum polycephalum*, is a classical material for studies of the structure of protoplasm and the mechanism of protoplasmic streaming. Observations of Marsland (12), Lewis (10), Camp (4), and others have shown that the protoplasm possesses a gel structure which may be reversibly transformed into a sol. The work reported in the present paper developed from considerations of this phenomenon and of how a cell might use it to transform chemical energy into mechanical work such as protoplasmic streaming.

*Physarum* has a shuttle type of protoplasmic streaming which changes direction rhythmically, the rate of flow ranging from zero to about 1.0 mm. per second (8). The motivating force, measured in terms of the hydrostatic pressure just sufficient to stop the protoplasmic flow, is of the order of 6 to 15 cm. of water (8). The flow is apparently powered by respiratory metabolism since agents such as dinitrophenol (DNP) which uncouple phosphorylative transfer from respiration inhibit streaming (18). This implies that the energy required for streaming is supplied as adenosinetriphosphate (ATP). The importance of ATP in streaming is more directly indicated by the findings of Goldacre and Lorch (7), and of Kriszat (9) that microinjection of ATP into ameba specifically increases the rate of protoplasmic flow. What then is the component of cytoplasm which responds to ATP? Loewy (11) has demonstrated that crude extracts of *Physarum polycephalum* plasmodia undergo reversible changes in viscosity in response to additions of ATP, and that inorganic phosphate is liberated in the process. In this respect, the behavior of the plasmodial extract is similar to that of an actomyosin preparation from muscle tissue.

The present paper concerns the nature of the ATP response of myxomycete protoplasmic extracts. Procedures for measuring the ATP response of the material are established. This information is then employed to develop a procedure for isolating the active component from the crude extract.

\* Report of work supported in part by a grant from the National Institutes of Health, United States Public Health Service.

‡ Contribution No. 2004.

### Materials and Methods

*Slime Mold Plasmodia.*—*Physarum polycephalum*<sup>1</sup> was cultured on oatmeal by a modification of the procedure of Loewy (11). A Petri dish wrapped with a paper towel is sprinkled with oatmeal and placed in a pan containing a shallow layer of water. The oatmeal is inoculated with sclerotia or with an inoculum from a growing culture, the pan is covered with a glass plate, then incubated in darkness or in low intensity light at 21 to 22°. In 48 to 72 hours the multinucleate plasmodia are collected from the water and pan surfaces, drained free of water, frozen, and stored at -10° until used.

*Extraction.*—The frozen plasmodia are shattered into small pieces and the fragments ground in a cold mortar at about 0° until a slush is formed. The extraction medium, generally 1.4 ml. per gm. of frozen plasmodia, is added to this slush. After 1 hour of extraction at -3 to 2°, the slurry is centrifuged (at 0 to 3°) in a SS-1 Servall centrifuge (90 v.) for 20 minutes. The turbid supernatant solution is decanted through a filter pad of glass wool to remove particles and coalesced lipid material, then centrifuged again at 20,000  $\times$  g for 20 minutes. The somewhat opalescent supernatant solution from the second centrifugation, the crude extract, usually contains about 1.5 per cent by weight of trichloroacetic acid (TCA) precipitable material.

*Test Solutions.*—Adenosinetriphosphate (ATP), adenosinediphosphate (ADP) (both Pabst Co.), and adenosinemonophosphate (AMP) (Ernst Bischoff Co.) were used without further purification. All other reagents were of reagent grade. All solutions were neutralized to pH 7.0 before use unless otherwise indicated in the text.

*Viscosity Determinations.*—Viscosities are determined in 4 ml. capillary viscometers with solvent flow times of about 60 seconds at 24.45°  $\pm$  0.03° and at maximum shear gradients of 1000 to 2000 sec.<sup>-1</sup>.

The relative viscosity,  $\eta_r$ , is expressed as the ratio of  $\rho_s t_s / \rho_o t_o$  in which  $\rho_s$  and  $\rho_o$  are the densities and  $t_s$  and  $t_o$  are the flow times of the solution and of the solvent respectively.

*Analytical Determinations.*—Protein concentration is determined by dry weight. Solutions are precipitated with two volumes of 1 N TCA at 0° and washed twice with three volumes of 0.5 N TCA. The washed precipitates are dried to constant weight in an oven at 100° (usually 72 hours) and the dry weight arbitrarily considered to be protein although, as shown by the anthrone test (14), some carbohydrate material is precipitated by the TCA.

ATPase activity is determined in the usual manner by the rate of release of inorganic phosphate from ATP. Conditions are chosen so that the reaction kinetics are first order with respect to protein concentration. Inorganic phosphate is determined by a modification of the Allen method (1).

### EXPERIMENTAL

*Extraction of Plasmodia with Buffered KCl Solutions.*—The influence of added ATP on the viscosity of a plasmodial extract depends greatly upon the extraction medium. Addition of 0.2 to 2.0  $\mu$ M of ATP per ml. to an extract prepared

<sup>1</sup> We are indebted to Professor W. Seifriz and Dr. A. Loewy for a gift of sclerotia to start our initial cultures.

with 1.4 M KCl-0.1 M phosphate buffer (pH 7.8 to 8.0) causes a small rise in viscosity (curve A, Fig. 1). A similar, though smaller, response to ATP is given by extracts prepared with 1.2 M KCl-0.1 M phosphate buffer solutions. This result is at variance with that of Loewy (11), who found that ATP produced sharp decreases in the viscosity of extracts prepared by the latter method.

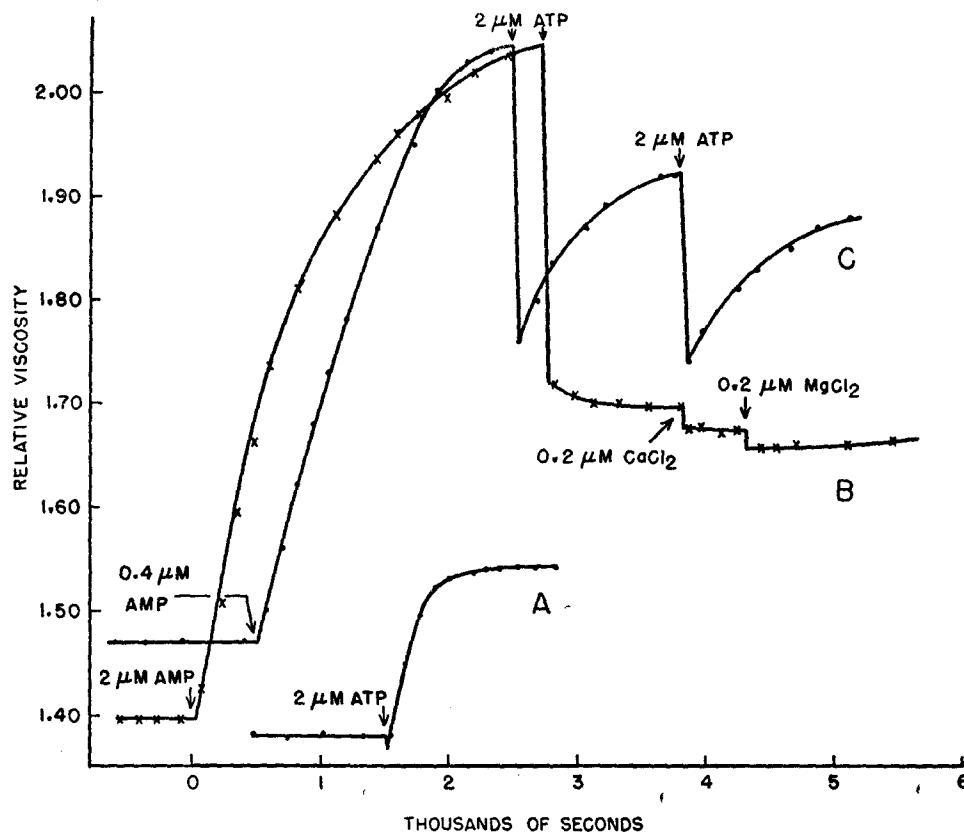


FIG. 1. Effect of ATP and AMP and of Ca and Mg ions upon the viscosity of 1.4 M KCl-0.1 M phosphate (pH 7.9) extract. Reagents added at times indicated by arrows. Concentrations are in micromoles per milliliter of extract.

Since extracts prepared at the higher salt concentration exhibited greater responses at a given protein concentration than did extracts prepared at low salt concentrations, extraction with 1.4 M KCl-buffer mixtures was employed as routine.

AMP in concentrations of 0.04 to 2.0  $\mu$ M per ml. of extract causes an immediate rise in viscosity, just as does ATP (curves B and C, Fig. 1). Subsequent addition of ATP to this system (after AMP) evokes a sharp reversible

decrease in viscosity (curve C, Fig. 1). Thus, before ATP can be effective in decreasing the viscosity of these extracts, the system must first be brought to a high viscosity level with AMP. The magnitude of the viscosity rise in response to AMP is a function of both AMP and protein concentration. Similarly, ATP-induced decrease in viscosity and recovery to a high viscosity level are functions of both ATP and protein concentration for any given concentration of AMP. The minimum concentration of AMP which evokes a noticeable rise in the viscosity of these crude extracts is  $0.04 \mu\text{M}$  per ml. while 10 times this amount elicits a near maximal response. Still higher concentrations ( $0.4$  to  $2.0 \mu\text{M}$  of AMP per ml.) produce slightly greater responses but are definitely inhibitory to the subsequent action of ATP, particularly to the recovery phase (curves B and C, Fig. 1). A minimum of about  $0.4 \mu\text{M}$  of ATP per ml. is required to affect the viscosity of extracts previously treated with submaximal amounts of AMP. Increasing the concentration of ATP from  $0.4$  to  $2.0 \mu\text{M}$  per ml. reduces the viscosity further but lengthens the recovery period and decreases the extent of recovery. An ATP concentration of  $2.0 \mu\text{M}$  per ml. causes a maximum reduction in viscosity, but in many instances the system fails to return to a high viscosity state, especially in the presence of more than minimal amounts of AMP. Addition of more AMP to a system brought to a low viscosity with excessive amounts of ATP does not again raise the viscosity, as it does in an original extract.

As noted above, a small rise in viscosity results when  $0.4$  to  $2.0 \mu\text{M}$  of ATP per ml. are added to crude extracts in the absence of AMP (curve A, Fig. 1). Subsequent addition of AMP ( $0.04$  to  $2.0 \mu\text{M}$  per ml.) to such ATP-containing solutions is without effect (compare with curves B and C, Fig. 1 in the absence of ATP). To be effective, AMP must be added prior to ATP. For example, when  $0.04 \mu\text{M}$  of AMP and  $0.4 \mu\text{M}$  of ATP per ml. are added simultaneously to the crude extract, only a small increase in viscosity results. This change is similar to that caused by addition of the same concentration of ATP alone to a crude extract (Fig. 2, compare with curve A, Fig. 1).

$\text{Mg}^{++}$  and  $\text{Ca}^{++}$  ions (as the chloride salts) in concentrations up to  $2 \mu\text{M}$  per ml., either individually or jointly, have no effect on the viscosity of the crude extract, the AMP or ATP response, nor on the recovery phase (curve B, Fig. 1).

The ATP effect on viscosity was used to compare systems extracted at various salt concentrations and acidities. This was done under conditions selected to yield maximal response to ATP, namely with a standardized submaximal concentration of AMP and a maximal concentration of ATP.

Of the systems extracted at pH 7.9 by  $0.6$ ,  $1.2$ , and  $1.4 \text{ M}$  KCl, that extracted at the highest salt concentration exhibits the greatest response to ATP (Fig. 3). When the extraction is carried out with  $1.4 \text{ M}$  KCl containing phosphate buffer at pH 6.7, 7.4, or 8.0, the system extracted at pH 8.0 is the most active.

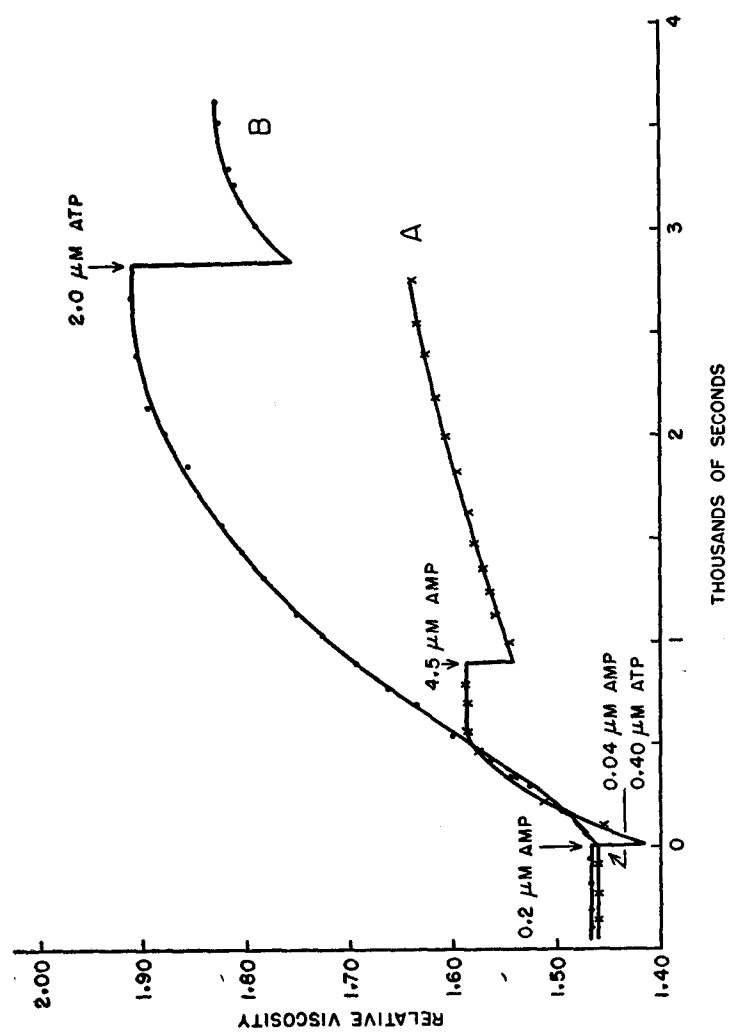


FIG. 2. Effect of AMP and of an AMP-ATP mixture upon the viscosity of a 1.4 M KCl-0.1 M phosphate (pH 7.9) extract.

Further, the system extracted with 1.4 M KCl at pH 8.0 exhibits greater changes in response to ATP and AMP when tested at pH 8.0 than at higher acidities. Substitution of 0.1 M THAM buffer at pH 8.4 or 0.1 M potassium maleate buffer

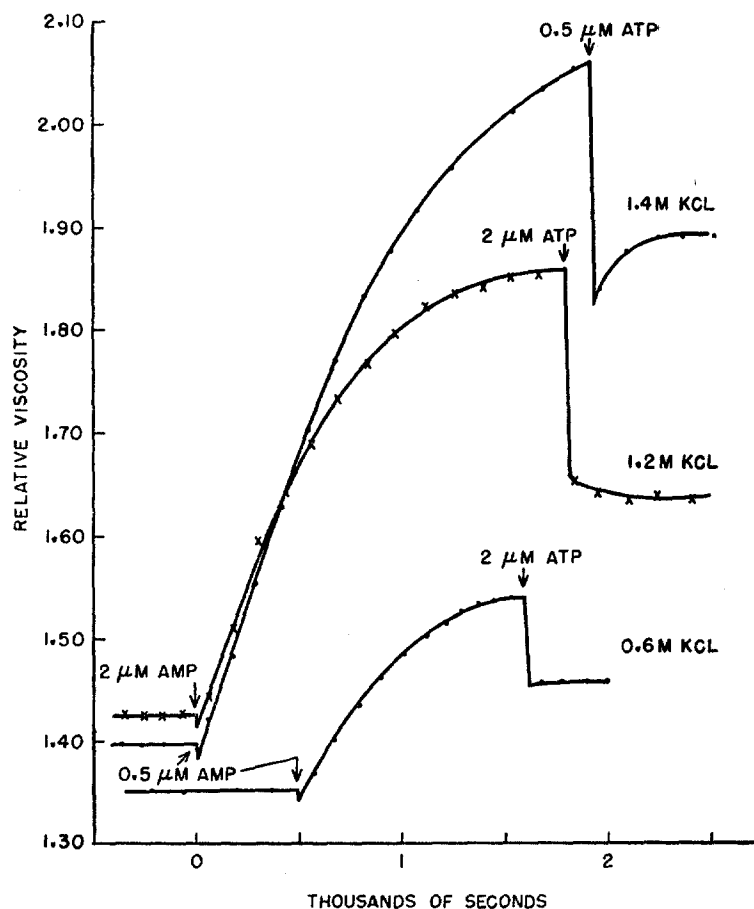


FIG. 3. Effect of maximal amounts of ATP upon the viscosity of solutions prepared by extraction with buffered (pH 7.9) KCl solutions at several KCl concentrations.

at pH 7.0 for the phosphate buffer in the extraction solution results in extracts which are completely inactive.

*Extraction with Unbuffered 1.4 M KCl.*—When the tissues of higher plants are disrupted, the large central vacuole may release acidic substances which denature the cytoplasmic proteins. Myxomycete plasmodia do not have a large central vacuole, hence neutral extracts of pH 6.9 to 7.0 are obtained when unbuffered KCl is used.

The properties of extracts prepared with unbuffered KCl differ from those prepared in the presence of phosphate ion. At equal protein concentrations, the initial viscosity of a 1.4 M KCl extract is less than that of a phosphate-buffered extract. ATP, AMP, or phosphate ion all raise the viscosity of KCl extracts. This effect is similar to that caused by AMP in extracts prepared with phosphate-buffered KCl. The threshold concentration and the maximum effective concentration are different for each compound. AMP is the most effective, with a threshold requirement of 0.02 to 0.04  $\mu\text{M}/\text{ml}$ . 0.15  $\mu\text{M}/\text{ml}$ . causes a relatively large increase in viscosity (curve A, Fig. 6).

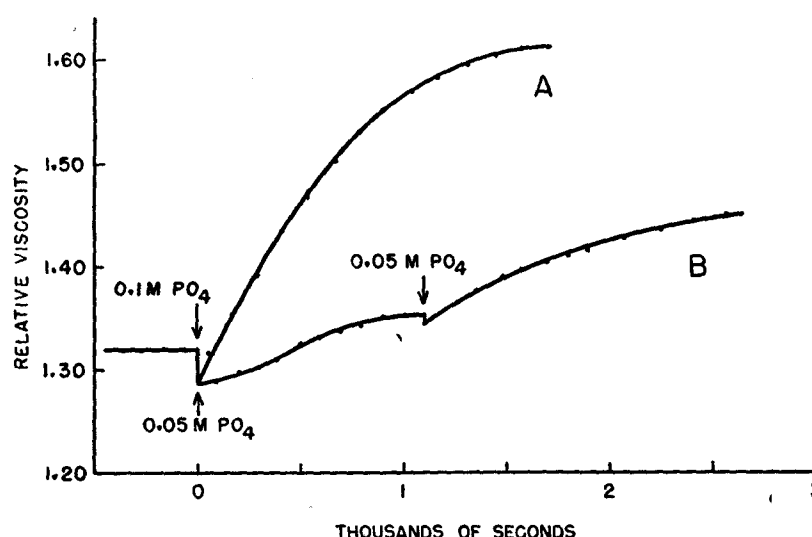


FIG. 4. Phosphate ion effect upon the viscosity of unbuffered 1.4 M KCl extracts. Protein concentration, *ca.* 10 mg./ml.

In the absence of AMP and phosphate ion, an initial addition of ATP causes an increase in viscosity of the 1.4 M KCl extract (curve A, Fig. 5), just as in the case of extracts prepared with phosphate-buffered KCl (curve A, Fig. 1). Subsequent additions of ATP temporarily lower the viscosity (curve A, Fig. 5).

Phosphate ion raises the viscosity of the unbuffered KCl extracts more than the other reagents tested, although the concentration required for maximum effect is higher. The response to phosphate ion is illustrated in Figs. 4, 5, and 6. Fig. 4 shows the effect of phosphate buffer (pH 7.0). 0.1 M phosphate is more effective than 0.05 M. Addition of more phosphate to the 0.05 M solution to make the final concentration 0.1 M raises the viscosity further, but not to the level caused by a single addition of phosphate to a final concentration of 0.1 M.

If pH 9.2 phosphate is added to a final pH of 8.0 and 0.1 M concentration,

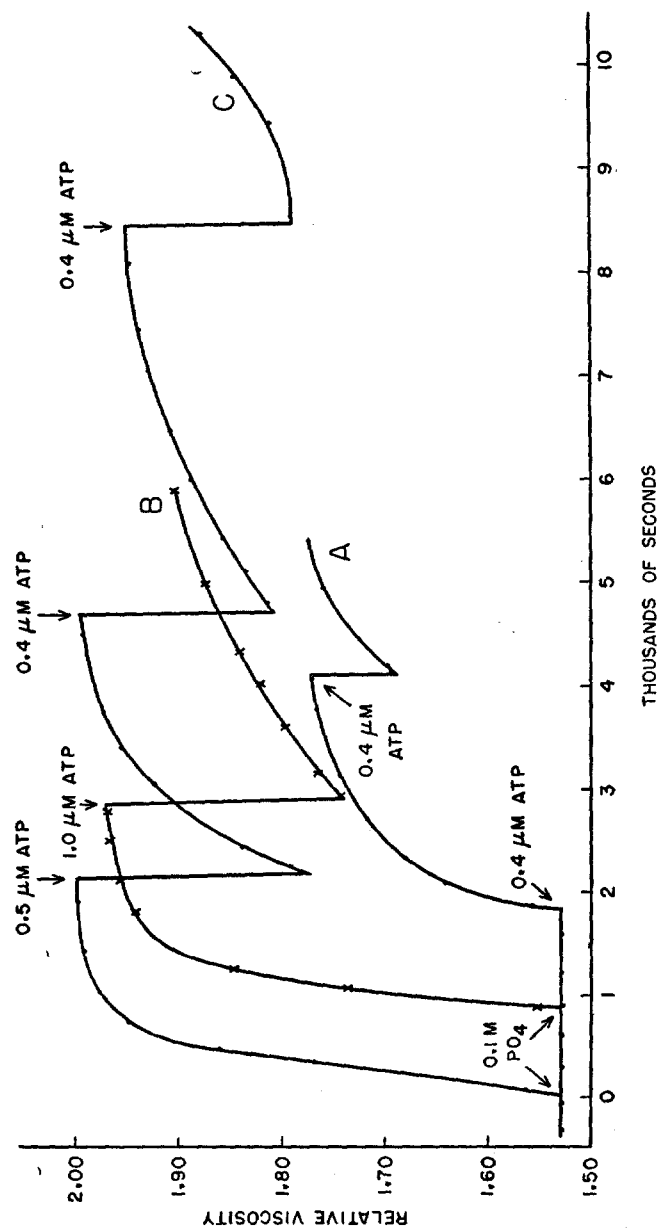


FIG. 5. Effect of phosphate ion and ATP upon the viscosity of an unbuffered 1.4 M KCl extract. Protein concentration, 4.5 mg./ml.



there is no change in viscosity of the preparation other than a small decrease due to dilution. Instead, precipitation of the protein occurs within 20 to 30 minutes.

Addition of AMP to KCl extracts previously made 0.1 M in phosphate ion causes a further small increase in the viscosity (curve C, Fig. 6). If prior to addition of phosphate, sufficient AMP is added to produce a maximal response, subsequent addition of up to 0.1 M phosphate causes little or no change in viscosity.

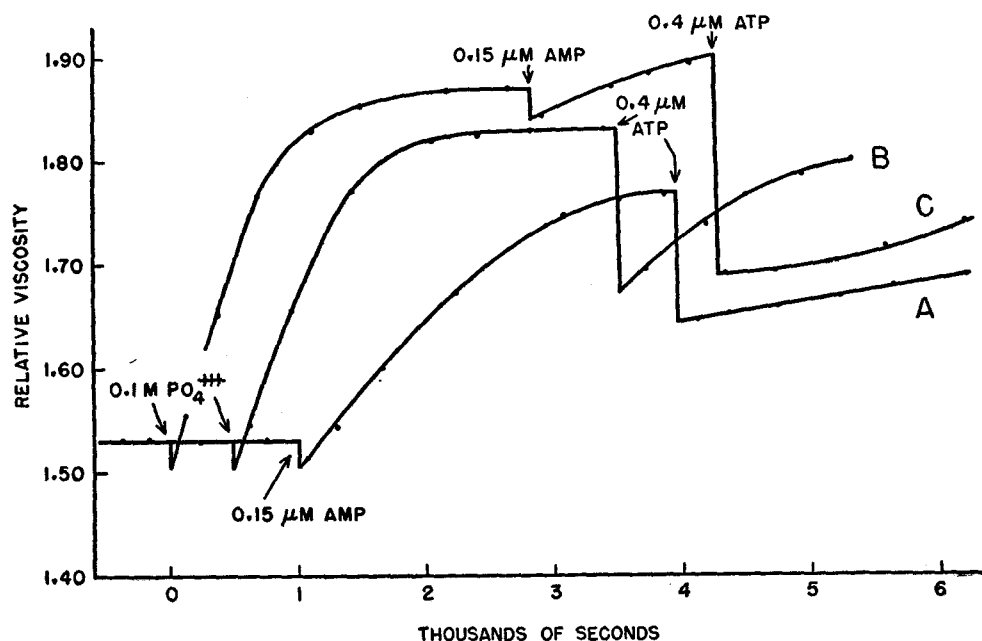


FIG. 6. Effect of phosphate ion, ATP, and AMP upon the viscosity of unbuffered 1.4 M KCl extracts. Protein concentration, 14.0 mg./ml.

Concentrations of ATP which cause small increases in the viscosity of KCl extracts lower the viscosity of solutions first brought to a high viscosity state with 0.1 M phosphate or with AMP (Figs. 5 and 6). Just as with systems extracted with KCl-phosphate buffer, the magnitude of the ATP response is a function of protein and ATP concentrations. An excessive ATP concentration causes a maximal drop in viscosity that is only slowly reversed. These solutions remain in a low viscosity state much longer than when lower ATP concentrations are employed. The concentration of phosphate ion has little effect on the extent of recovery following addition of ATP, but if AMP is used to raise the viscosity prior to addition of ATP, the AMP tends to inhibit recovery of the system (compare curves A and C with curve B, Fig. 6).

The specificity of the system constructed by addition of 0.1 M phosphate to the 1.4 M KCl extract has been tested briefly. Adenosine is without effect. ADP causes a smaller drop in viscosity than does a similar concentration of ATP, but recovery is considerably slower. Sodium tripolyphosphate has no effect on a system initially in either a low or high viscosity state. Sodium pyrophosphate causes precipitation of protein within a few minutes.

Unbuffered KCl extracts lose all of their ability to respond to phosphate, AMP, or ATP, if they are dialyzed against 1.4 M KCl or buffers overnight. Nearly all of the activity is lost if the preparations are stored at 0° for a similar period of time. Approximately half of the activity is lost if the material is frozen and stored at -10° for a week.

In summary, KCl extracts of plasmodia exhibit marked changes in viscosity in response to addition of adenylates. The extracts are in a low viscosity state as prepared. AMP, inorganic phosphate or, under certain conditions, ATP raise the viscosity of these extracts. ATP, when added in suitable amounts, temporarily lowers the viscosity; this cycle can be repeated.

*(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> Fractionation of the Unbuffered 1.4 M KCl Extract.*—When the 1.4 M KCl extract is fractionated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (SAS) at pH 7.0 and 0°, only the fraction precipitated between 30 and 40 per cent saturation has the ability to change viscosity in response to ATP. All the fractions redissolve readily in KCl of any concentration or in buffers. In the experiments described below, the precipitates have been redissolved in 1.4 M KCl unless otherwise indicated.

The initial viscosity of the 30-40 SAS fraction is high in comparison with that of the crude KCl extract, the 0 to 30 per cent SAS fraction, or the supernatant which remains after 40 per cent saturation. Addition of 0.2 to 1.6 μM ATP/ml. rapidly reduces the viscosity, which then recovers spontaneously. This process can be repeated (Fig. 7). The threshold amount of ATP required to produce a measurable change in viscosity is lower than that required to produce a similar response in the unfractionated extract. Further, although the effect per micromole of ATP is greater here than in the unfractionated crude extracts, the recovery phase is suppressed by an excess of ATP. For example, a single addition of 3 μM of ATP per ml. of solution is sufficient to keep a salt-fractionated preparation in a low viscosity state for several hours. To keep unfractionated preparations in the low viscosity state, repeated additions of similar amounts of ATP are necessary. Only ATP changes the viscosity of salt-precipitated preparations. AMP and ADP are without effect. Inorganic phosphate is no longer required to bring the purified preparations to a high viscosity state. It has not been possible to demonstrate any effect of Mg<sup>++</sup> or Ca<sup>++</sup> ions on the viscosity of the salt-precipitated material.

Total phosphorus (TP), trichloroacetic acid-precipitable phosphorus (TCA-P), and ATPase and phosphatase activity were each measured in the crude extract and in each fraction of several preparations. Representative re-

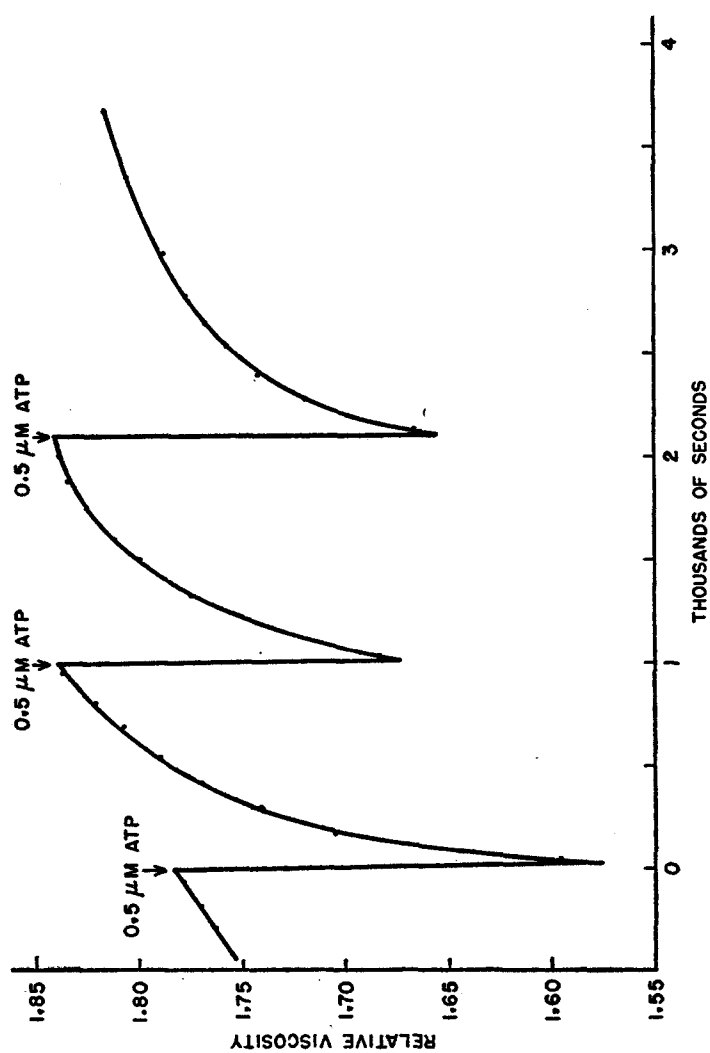


Fig. 7. Response of 30-40 SAS fraction to ATP.

sults are summarized in Table I. The active fraction contains a substantial portion of the TCA-P of the whole extract. On the other hand, most of the phosphatase activity and the conventional ATPase activity has been removed. There is, however, associated with the purified fraction an ATPase activity which acts specifically in connection with viscosity changes induced by ATP. The recovery phase is accompanied by release of inorganic phosphate from ATP (Fig. 8).

Preparations of salt-fractionated materials exhibit several interesting relationships between thermal history and viscosity. These depend upon concentration, rate of shear, and amount of working. Surprisingly, chilling the samples

TABLE I  
*Summary of Properties of  $(\text{NH}_4)_2\text{SO}_4$ -Precipitated Fractions\* of 1.4 M KCl Extracts*

Fraction	Relative viscosity of 1.5 per cent protein solution	ATP response	Protein	Total phosphorus	TCA-P	Relative activity per mg. protein	
						ATPase	Phosphatase
Initial extract	1.6 to 1.8	++	per cent 100	per cent 100	per cent 100	100	100
0-32 per cent SAS precipitated	1.1 to 1.15	None	10-15	5	8	31	30
32-40 per cent SAS precipitated	2.6 to 3.4	++++	25-30	42	57	16	33
40 per cent SAS supernatant solution	1.1 to 1.2†	None‡	55-60	50	18	95	179
Recovery			90-95	97	83		

\* Precipitated fractions redissolved in 1.4 M KCl.

† Measured in 40 per cent SAS.  $(\text{NH}_4)_2\text{SO}_4$  *per se* does not affect ATP response or viscosity of other fractions when present below concentrations which cause incipient precipitation.

lowers the viscosity and subsequent warming raises it to the initial or even to a higher value. Many of these solutions, particularly those with higher than initial viscosities, work-soften; *i.e.*, become less viscous, as a result of flow through the viscometer capillary.

It is convenient to regard the total viscosity of these solutions as being made up of two contributing viscosities: first, the normal viscosity of a solution containing elongated or highly hydrated solutes, and second, a structural viscosity resulting from formation of loose aggregates of the solute. It is the structural viscosity contribution which is increased as a result of warming the solution following a chilling treatment and which is responsible for the work-softening behavior. The increased structural viscosity may be destroyed by sufficient working of the solution, by chilling, or by ATP. Once the structure has been

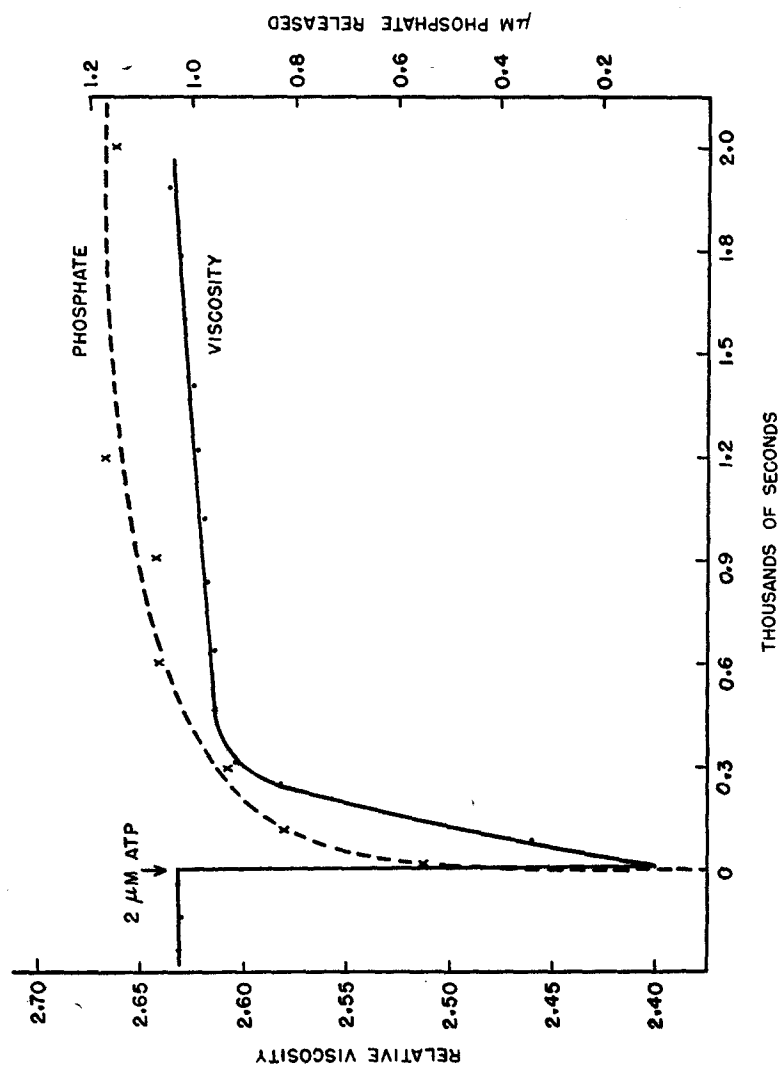


FIG. 8. Relationship between the ATP-induced change in viscosity and rate of release of inorganic phosphate ion in 30-40 SAS fraction.

broken by mechanical action, it is not regenerated, although it is if destroyed by chilling and is at least partially if destroyed by ATP.

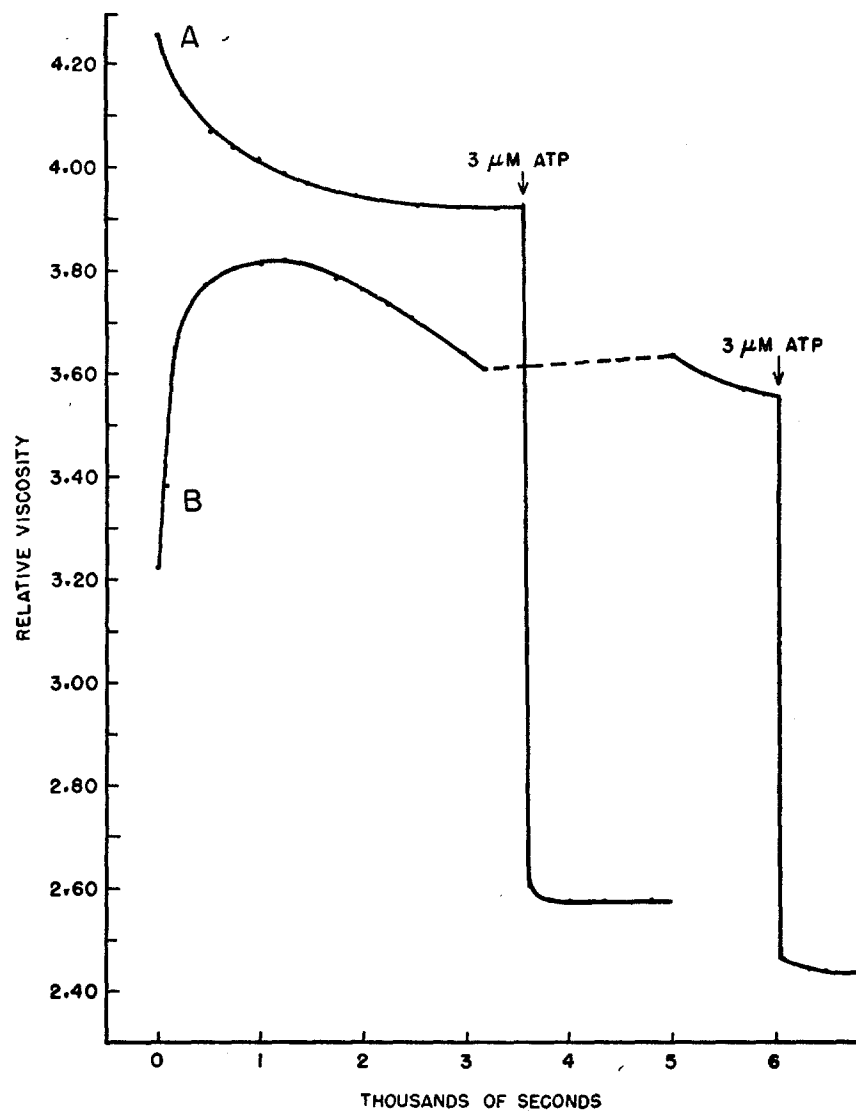


FIG. 9. Work-softening and thermal history effects upon the viscosity of the 30-40 SAS fraction. See text for details. Protein concentration, 24.4 mg./ml.

Regeneration of structural viscosity following cold treatment and subsequent work-softening is illustrated in Fig. 9. A solution containing 24.4 mg./ml. protein was divided into two parts immediately after preparation. Solution A

was placed in a viscometer, equilibrated for 13 minutes, measured once, then stored for 3.25 hours in the viscometer bath. At end of this time, the viscosity was unchanged. Solution B was stored at 0° for 3.25 hours, then placed in a viscometer, and both solutions measured repeatedly without waiting for the chilled sample to reach temperature equilibrium. The viscosity of solution A decreased during successive measurements<sup>2</sup> and appeared to approach a limiting value. Before solution B warmed up, its viscosity was lower than that of solution A, but on warming it the viscosity approached that of solution A. However, as successive measurements were made, part of this generated viscosity broke down. The dotted portion of curve B shows that when the solution is not worked for an extended period of time the viscosity remains essentially constant. This demonstrates that: (a) the decrease in viscosity during successive measurements is work-softening and depends on working and not on time; (b) once the structural viscosity is broken down by working the solution, it is not regenerated under the conditions of these experiments. That is, the solution is not thixotropic. Subsequent addition of excess amounts of ATP (from which there is no recovery) reduced both solutions to a low, and essentially equal, viscosity.

The effect of ATP on the structural viscosity induced by prior thermal treatment is shown in Fig. 10 for a more dilute preparation (15.5 mg./ml.). Solutions A, B, and C are portions of the same solution. This preparation initially had a relative viscosity of 2.65, and showed no work-softening. Solutions B and C were stored for 2 hours at 0°, and then stored in viscometers at 24.5° for 40 minutes. Solution A was stored at 24.5° (no cold treatment). As shown in Fig. 10, the chilling and subsequent warming of solutions B and C raised the viscosities from 2.65 to 3.35, and induced work-softening. In contrast, the relative viscosity of solution A did not change with working. Addition of 0.5  $\mu$ M of ATP/ml. to solution A caused a nominal decrease in viscosity. Recovery was rapid and complete. A second addition of the same quantity of ATP produced a greater effect and recovery, which was incomplete, was much slower. Addition of 0.5  $\mu$ M of ATP/ml. to solution C, which shows structural viscosity, lowered the viscosity more than did the same amount of ATP in solution A. Equivalent amounts of ATP, however, did not reduce the viscosity of both solutions to the same level.

The decrease in viscosity is not proportional to ATP concentration.<sup>3</sup> (Compare Figs. 10 B and 10 C.) Furthermore, the higher the ATP concentration used, the slower and less complete the recovery. In both of these solutions, suf-

<sup>2</sup> In contrast, the viscosity of similar solutions at lower protein concentrations (*ca.* 10 mg./ml.) and hence at correspondingly lower viscosity values, remained constant even when measured frequently over a 6 hour period.

<sup>3</sup> There is a ground level of viscosity in the preparations which cannot be lowered by any amount of ATP. This is a function of the protein concentration, and in the region of *ca.* 25 mg./ml. it is reached at an ATP concentration of *ca.* 3 to 4  $\mu$ M/ml.

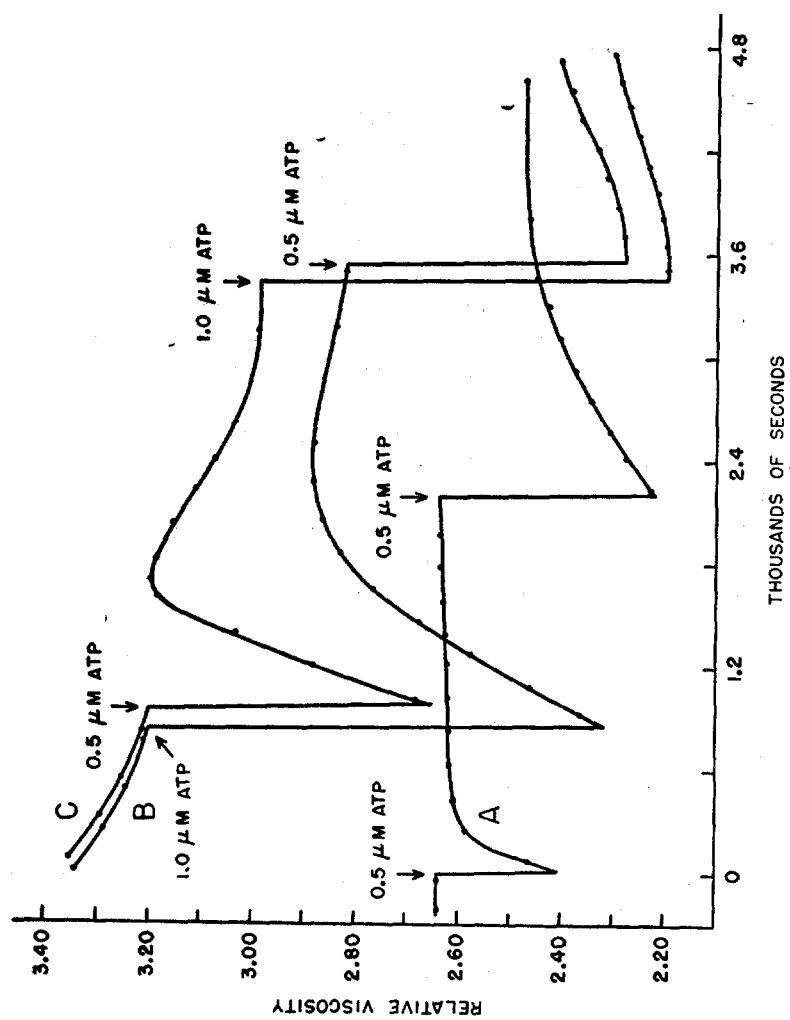


FIG. 10. Destruction by ATP of the structural viscosity generated by low temperature treatment of the 30-40 SAS fraction. See text for details. Protein concentration, 15.5 mg./ml.



ficient structural viscosity was regenerated following one addition of ATP so that the solutions exhibited work-softening. Solution A, which did not have a cold treatment and did not work-soften initially, did not exhibit work-softening after recovery from ATP.

Throughout the development of an isolation procedure, the instability of the crude extracts and of the purified fraction has been a serious handicap. Preparations stored at 25° lose ability to respond to ATP, although not viscosity, in 3 to 12 hours, depending upon the degree of purification. Those stored at 0° lose about 50 per cent of their activity in 24 hours. Freezing of either the crude extract or of the 30–40 SAS fraction generates structural viscosity, and if solutions are kept frozen longer than 18 to 24 hours, considerable material may not go back into solution when thawed. Dialysis for 12 to 18 hours at 0° against any concentration of KCl or a number of different buffers always results in complete loss of activity and degeneration of the viscosity. Addition of 0.1 to 0.5  $\mu$ M of ATP per ml. of external solution during dialysis preserves some of the viscosity and activity but not enough to be useful. Addition of  $Mg^{++}$  or  $Ca^{++}$  ions to these dialyzed solutions is without effect.

If the 30–40 SAS precipitate is stored at 0° for 6 to 24 hours before it is redissolved, it retains all of its activity. Solutions made from the stored precipitate have the same rheological properties as if they had been prepared immediately. The same is true if the precipitate is frozen and stored at –8° for 3 or 4 days. This provides a convenient point to break an otherwise lengthy preparative procedure and a method of preserving, reproducibly, portions of material for subsequent analysis.

When the ammonium sulfate-precipitated fraction is taken up in 0.2 M KCl instead of 1.4 M KCl, the viscosity of the solution at 24.5° is not affected by storage at 0° or by freezing. No structural viscosity is generated. Furthermore, these solutions may be dialyzed at 0° for 10 to 12 hours against 0.1 M KCl or 0.1 M KCl + 0.1 M potassium maleate buffer at pH 7.0 without loss of either viscosity or the ability to respond to ATP. All these properties are in contrast to those discussed above for the solutions in 1.4 M KCl. This made continuation of the isolation procedure feasible, for it is possible to extract at high salt concentrations, precipitate the active material with  $(NH_4)_2SO_4$ , and store the precipitate until needed. Lastly, physical methods may be used to characterize the various fractions because the salt concentration may be sufficiently reduced for electrophoresis and the solvent may be specified for viscosity experiments and ultracentrifuge analyses.

*Properties of the Slime Secreted by the Plasmodia.*—The myxomycetes are commonly called slime molds because of the mucous-like slimy material secreted by the plasmodia. This material is largely carbohydrate. Since no precautions are taken to exclude all of the slime material during harvest, the plasmodia contain varying amounts of carbohydrate which is in part extracted

during the first step in the preparative procedure. Indeed, analyses by the anthrone reagent method (14), show that in active salt-precipitated fractions as much as 16 to 20 per cent of the total soluble material and about 10 per cent of the total TCA-precipitable material is carbohydrate. Since high molecular weight carbohydrates form viscous solutions and since the ether linkage in polyethers such as methyl cellulose is one of the few types of bonds known to have a positive temperature coefficient of viscosity, the slime material has been cursorily examined with respect to its extractability, viscosity, response to ATP, and to thermal history.

Under the culture conditions employed, the plasmodia of *Physarum* grow on the surface of the water and secrete large amounts of slime into the water. This slime is substantially clear and forms thick jelly-like masses that may be harvested almost free of the yellow strands of plasmodia. After several days of growth, the plasmodial strands cover the moist sides of the culture tray and the underside of the glass cover. These strands, particularly those on the cover, appear to be free of slime, but the quantity of material is limited.

Slime material nearly free of plasmodial material was extracted with unbuffered 1.4 M KCl. The resulting extracts were found to have a high viscosity but to show no change in viscosity in response to ATP or inorganic phosphate ion. They do not exhibit any of the thermal history effects characteristic of the 1.4 M KCl extracts of plasmodia, as described above. When the 1.4 M KCl extracts of the slime material are fractionated with  $(\text{NH}_4)_2\text{SO}_4$ , the distribution of precipitable material vs. salt concentration differs from that of the plasmodial extracts. All the fractions obtained are highly viscous but none exhibits any response to ATP nor do they show thermal history effects.

Extraction and fractionation of plasmodia which are as free of slime as possible yield results identical with those from plasmodia containing considerable quantities of slime. Thus, the carbohydrate apparently is not a necessary part of the ATP-responsive system. The effect, if any, of the carbohydrate is to increase the ground level viscosity of the active fraction.

#### DISCUSSION

The fact that there is a material in myxomycetes which undergoes reversible changes in physical properties in response to ATP raises two questions. First, what is the physiological role of this material in the plant cell? Second, what is the molecular nature of the ATP-induced change in the material itself?

Two cellular processes, other than enzymatic syntheses, which appear likely to involve ATP-protein interaction are the active adsorption of solutes (osmotic work) and protoplasmic streaming. Both processes are dependent upon the supply of ATP produced by oxidative metabolism. In the presence of uncoupling agents of oxidative phosphorylation, such as 2,4-dinitrophenol (DNP), both osmotic work and streaming stop.

Active uptake of water and of ions presumably involves a change in hydration and/or charge of some carrier protein molecule. Cytologists have also postulated a cytoplasmic matrix which surrounds and supports the particulate structures of the cell. It has been suggested that this matrix may participate in protoplasmic streaming by undergoing a reversible rhythmic sol-gel transformation (*e.g.* Marsland, (12)). An ATP-induced change in viscosity, taken by itself, is only indicative of a change in the size and shape of the effective hydrodynamic unit. Such a change may be caused by a change in hydration, a change in molecular charge (which in turn may change the hydration or cause aggregation or disaggregation of molecular units), or a change in aggregate or monomer size. Thus, no conclusive deduction in regard to a possible physiological role of the protein studied here may be made from the viscosity data presented.

No further data in regard to the possible role of the ATP-sensitive protein in active adsorption are available, but there is indirect evidence to implicate it in protoplasmic streaming.

Myxomycetes do not have a rigid cell wall. The protoplasm is enclosed by a plasmalemma and surrounded by a slimy, structureless, colorless material, the sheath. The protoplasm itself is differentiated into the plasmasol, or liquid cytoplasm, which streams through channels in the plasmagel, the gelled portion of the cytoplasm. The plasmagel and plasmasol undergo localized gel-sol transformations as pseudopodia are formed and withdrawn at the advancing edge of a plasmodium (Andersen and Pollock (3), Marsland (12), and Camp (4)). Inclusion bodies (nuclei, vacuoles, pigment bodies, and mitochondria) are distributed randomly through both plasmagel and plasmasol.

As indicated above, streaming is an energy-requiring, ATP-dependent phenomenon. Microinjection of low concentrations of neutral ATP solutions into plasmodia partially liquifies the plasmagel and increases the rate of streaming. Within a few seconds to a few minutes, protuberances start to form randomly on the periphery of the plasmodium. Similar results are obtained if the ATP is introduced into the strand by diffusion. The time course of formation and the cytology of these protuberances vary with the ATP concentration. At low ATP concentrations, the protuberances resemble pseudopodia although they are formed at any point on the plasmodium rather than at the advancing tip. Higher concentrations of ATP cause much of the plasmagel to be liquified. Organized streaming stops, formation of the protuberances is rapid, and high internal pressures are developed in the plasmodium. Soon one or more of the protuberances ruptures, leaving a definite membrane-like shell. The cytoplasm leaks out. Concurrently, expansion of other protuberances on the same strand stops or the protuberances may even shrink somewhat in size as the pressure is released. The effects of ATP on myxomycete plasmodia are similar in many respects to those recently reported by Goldacre (6, 7) for *Ameba*.

The gel-sol transformation seems to be specific for ATP although both ATP and sodium triphosphate cause the formation of protuberances on the strands. Orthophosphate, ADP, and AMP are all without effect. Immersion of plasmodia in dilute solutions of detergents, organic solvents, acids, and alkalis kills the protoplasmic strand but without formation of protuberances or the accompanying intrastrand changes described for ATP (17).

Immersion of plasmodial strands in  $1 \times 10^{-3}$  M DNP stops protoplasmic streaming within 5 minutes. In  $2 \times 10^{-4}$  M DNP, streaming gradually slows down, then stops completely in 30 to 40 minutes. When streaming stops, the strands remain intact but appear to be dead. Streaming does not commence again after the strands are immersed in water.

The fact that injection of ATP liquifies the plasmagel, decreases the viscosity of the plasmasol, and increases the rate of protoplasmic streaming *in vivo*, plus the fact that we have extracted a protein which exhibits similar ATP-induced reversible changes in viscosity *in vitro* suggests that the extracted material may be functional in the streaming phenomenon.

From a physical chemical standpoint, the ATP-sensitive protein presents several interesting problems. Specificity studies and interpretation of the effects of AMP, ADP, ATP, and orthophosphate upon the viscosity are difficult because of rapid enzymatic interconversion of these reagents. Either AMP or phosphate ion is required to raise the viscosity of KCl extracts before ATP can be effective. The action of ADP and of an initial portion of ATP (as in curve A, Fig. 5) in increasing the viscosity may be attributed to enzymatic degradation of these reagents to AMP. Similarly, the effect of ADP in decreasing the viscosity of preparations previously made more viscous by AMP may be attributed to the conversion of ADP to ATP by a myokinase system.

The way in which AMP and orthophosphate act to increase the viscosity of crude 1.4 M KCl extracts is unknown. The increase in viscosity itself is most readily interpreted as an aggregation process, particularly in view of the behavior of salt-fractionated preparations which exhibit aggregation and which do not require AMP or inorganic phosphate. Indeed, the disparity in the concentrations of AMP and of orthophosphate required for maximum response is so great that the mechanism(s) through which they exert their effect may be entirely different although the end result, an apparent aggregation, may be the same.

Further work upon the crude extracts is indicated because of their inherent interest. These extracts, which represent a close approach to a native cytoplasm, possess a definite physiological response *in vitro*. Work with these preparations is difficult, however, because, although they do not exhibit work-hardening, work-softening, or thermal history effects, quantitative reproducibility between various preparations is not good. Furthermore, because

enzymatic activity of the extracts is so high, rapid autolysis occurs even at 0°. Preparations lose much of their ability to respond to primers or to ATP within hours. This makes storage or dialysis impossible.

The results obtained from salt-fractionated preparations have been more definitive. First, no priming effect is observed with AMP or orthophosphate. The 30–40 SAS preparations have a high initial viscosity which is decreased by ATP. These preparations, however, are complicated by work-softening and thermal hardening effects which make prior thermal and work histories important in interpreting experimental data.

The thermal history and work-softening observations are consistent with the hypothesis that the ATP-sensitive material undergoes a reversible aggregation (in the presence of primer in crude extracts or spontaneously in salt-fractionated preparations) to form viscous solutions. The aggregation process has a positive temperature coefficient. Hence, on cooling disaggregation occurs. This accounts for the thermal history effects observed in purified preparations. This type of behavior is fairly common in polymer chemistry and in biological systems. Costello (5), for example, has reported that the cytoplasm of *Arbacia* eggs becomes less "viscous" as the temperature is decreased and Mirsky (13) has extracted a protein from *Arbacia* eggs which becomes less viscous as the temperature is lowered or pressure increased. More recently somewhat analogous association processes with a positive temperature coefficient have been reported by Parrish and Mommaerts for actomyosin (15), Allison, Murayama, and Vinograd for sickle-cell hemoglobin (2), and Schachman for bushy stunt virus following partial enzymatic digestion (16).

Work-softening is indicative of breakdown of the postulated aggregates as a result of stress imposed by shear gradients in the viscometer capillary tube. If this breakdown process were merely the rupture of bonds formed during thermal hardening, then healing should occur as rapidly as the initial thermal hardening, which is a fast process. Since healing does not occur, the bonds broken as a result of mechanical stress are probably not the same as those ruptured by thermal conditioning at low temperature.

The function of ATP within the framework of the above hypothesis is to break down the aggregates which form spontaneously in purified preparations.

#### SUMMARY

1. Extracts of the plasmodia of the myxomycete, *Physarum polycephalum*, exhibit reversible decreases in viscosity in response to the addition of ATP under appropriate conditions. The protoplasm material prepared by extraction with KCl solution can apparently exist in either a high or a low viscosity state. As prepared, it is in the low viscosity condition. Rapid and extensive increases in viscosity of the extract are brought about by addition of AMP,

inorganic phosphate, or, under certain conditions, of ATP. Only after the high viscosity state has been attained does addition of appropriate quantities of ATP cause a reversible decrease in viscosity.

2. The active principle of crude plasmodial extracts may be concentrated by fractional precipitation with ammonium sulfate and is found in the fraction precipitated between 30 and 40 per cent saturation. This material possesses a higher viscosity than does the original crude extract and is apparently in the high viscosity state since the addition of ATP causes an immediate reversible decrease in viscosity.

3. The ATP-sensitive fraction of myxomycete plasmodia possesses a viscosity which is dependent upon its previous thermal treatment. Extracts incubated at 0° for a period of a few hours increase greatly in viscosity when they are returned to 24.5°. This increased viscosity is structural in nature, is destroyed by mechanical agitation of the solution, and may be reversibly destroyed by addition of ATP.

4. It is suggested that the ATP-responsive protein of myxomycete plasmodia may be related to sol-gel transformations which have been observed in intact plasmodia and may participate in the protoplasmic streaming of the intact organism. This suggestion is based upon the following facts: (a) the protoplasmic streaming of myxomycete plasmodia is increased by microinjection of ATP; (b) the gel portion of the cytoplasm at the site of the microinjection of ATP is extensively converted to the sol state.

The changes in structure of the intact cytoplasm are thus similar in nature to the changes exhibited in response to ATP by the purified ATP-sensitive protein.

5. The ATP-sensitive protein of myxomycete plasmodia appears to undergo reversible aggregation to form a high viscosity state. The function of ATP is to break down the aggregates thus formed. Since a specific ATPase activity is associated with the purified material, added ATP is gradually destroyed and recovery of viscosity attends the spontaneous reconstitution of aggregates.

#### REFERENCES

1. Allen, R. J. L. *Biochem. J.*, 1945, **34**, 358.
2. Allison, A. C., Murayama, M., and Vinograd, J., personal communication.
3. Andersen, N. and Pollock, B. M., *Compt. rend. Lab. Carlsberg., Ser. chim.*, 1952, **28**, 247.
4. Camp, W. G., *Bull. Torrey Bot. Club*, 1937, **64**, 307.
5. Costello, D. P., *J. Cell. and Comp. Physiol.*, 1934, **4**, 421.
6. Goldacre, R. J., *Internat. Rev. Cytol.*, 1952, **1**, 135.
7. Goldacre, R. J., and Lorch, I. J., *Nature*, 1950, **166**, 497.
8. Kamiya, N. Physical aspects of protoplasmic streaming, *in* The Structure of Protoplasma (W. Seifriz, editor), Ames, Iowa State College Press, 1942, 237.

9. Kriszat, G., *Ark. Zool.*, (2), (1950) **1**, 477. *Chem. Abstr.*, 1951, 10409 h.
10. Lewis, W. H. The relation of viscosity changes in protoplasm to ameboid locomotion and cell division, *in* The Structure of Protoplasm, (W. Seifriz, editor, Ames, Iowa State College Press, 1942, 163.
11. Loewy, A. G., *J. Cell. and Comp. Phys.*, 1952, **40**, 127.
12. Marsland, D. A. Protoplasmic streaming in relation to gel structure in cytoplasm, *in* The Structure of Protoplasm, (W. Seifriz, editor), Ames, Iowa State College Press, 1942, 127.
13. Mirsky, A. G., *Science*, 1936, **84**, 333.
14. Morris, D. L., *Science*, 1945, **107**, 254.
15. Parrish, R. G., and Mommaerts, W. F. H., *J. Biol. Chem.*, 1954, **209**, 901.
16. Schachman, H. K., personal communication.
17. Ts'o, P. O. P., Thesis, California Institute of Technology, 1955.